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Concise Review: Modeling Neurodegenerative Diseases with Human Pluripotent Stem Cell-Derived Microglia

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Key Words. Experimental models • Induced pluripotent stem cells • Myeloid cells • Yolk sac cell • Neuroimmune • Glia

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ABSTRACT

Inflammation of the brain and the consequential immunological responses play pivotal roles in neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and frontotemporal dementia (FTD). Microglia, the resident macrophage cells of the brain, have also emerged as key players in neuroinflammation. As primary human microglia from living subjects are normally not accessible to researchers, there is a pressing need for an alternative source of authentic human microglia which allows modeling of neurodegeneration in vitro. Several protocols for induced pluripotent stem cell (iPSC)-derived microglia have recently been developed and provide unlimited access to patient-derived material. In this present study, we give an overview of iPSC-derived microglia models in mono-culture and coculture systems, their advantages and limitations, and how they have already been used for disease phenotyping. Furthermore, we outline some of the gene engineering tools to generate isogenic controls, the creation of gene knockout iPSC lines as well as covering reporter cell lines which could help to elucidate complex cell interaction mechanisms in the microglia/neuron coculture system, for example, microglia-induced synapse loss. Finally, we deliberate on how said cocultures could aid in personalized drug screening to identify patient-specific therapies against neurodegeneration. *STEM CELLS* 2019;00:1–7

SIGNIFICANCE STATEMENT

Microglia and neuroinflammation are at the center in current research efforts to identify targets to fight various neurodegenerative diseases. However, the tragic failures of many recent drug candidates in clinical trials showed that the results from mouse models have only limited transferability to the clinical setting. Human iPSC-derived neuron/microglia cocultures emerge as an authentic human, pre-clinical tool, for drug candidate validation. Testing a series of drugs on big patient cohorts will allow substratifying of patients into drug responders and nonresponders whereas this study foresees a future where complex patient-specific cocultures are used in personalized medicine.

INTRODUCTION

Microglia are the resident macrophages of the brain. Most models of neurodegenerative diseases used in preclinical research are murine models which allow for the study of microglia either directly within the brain, in organotypic slice cultures, or as primary microglia upon isolation from fresh brain tissue or from ex vivo cocultures with astrocytes. From the various available mouse disease models, there is, therefore, theoretically, unlimited availability of primary or cultured mouse microglia. The drawback, however, is the limited translation of mouse results to human pathologies, seen because of the differences in the key modulators of their respective neuroinflammatory pathways [1] and differential expression of risk genes [2].

A meaningful analysis of freshly isolated microglia from healthy or patient's brain is extremely difficult since the material is very limited and the tissue obtained post-mortem or during brain surgery often comes with artifacts or comorbidities. Most research in human microglia is, therefore, done with cultured fetal or adult primary microglia. These cultures although have been shown not to be a very authentic model for the microglia in the brain environment and loose microglial transcriptome signature within the first hours ex vivo culture [3].

Brain samples of patients with neurodegenerative disease are extremely rare and normally only accessible post-mortem. However, in order to understand the initiation of pathology or for screening for potential drugs that reduce the pathology, material from presymptomatic

patients, or samples from early stages of the disease would be most valuable. Induced pluripotent stem cells (iPSCs) can be derived from many easily accessible tissue sources of patients such as skin fibroblasts or blood. Many protocols have been developed to differentiate iPSCs to the key players in neurodegeneration [4] (i.e., neurons, microglia, astrocytes, and oligodendrocytes) and for adult onset neurodegenerative diseases, we can expect that in many cases those iPSC-derived cell types represent the presymptomatic stage of the disease displaying physiological deficits preceding neuronal death. Furthermore, it is known that maturing iPSC to adult neurons takes more than 100 days of differentiation [5].

Blood monocytes are easily accessible, and protocols exist that claim to skew them toward a microglia identity [6–8]. Later transcript analysis, however, revealed that these blood monocyte-derived microglia differ significantly from microglia in the brain [9]. Adult blood monocytes derive from myeloblasts proto-oncogene, transcription factor (MYB)-dependent hematopoietic stem cells (HSCs) in the bone marrow, whereas microglia originate from MYB-independent yolk sac derived fetal macrophages that invade the human brain around embryonic day 31 until closure of the blood–brain barrier (BBB) proliferate locally and are not replaced by other macrophages in healthy individuals [10]. Therefore, blood monocyte-derived microglia are likely to resemble the blood monocyte-derived cells found in the brain after stroke. These cells adapt microglial morphology but differ both functionally and at transcriptome level from resident microglia [11]. Therefore, we believe that, in order to obtain a near-authentic microglia model, one should faithfully mimic the microglial ontogeny and provide a neuronal environment. The cells should differentiate in a MYB-independent manner to yolk sac-derived fetal macrophages and be allowed to invade into a neuronal environment, where they mature and adapt ramified microglial morphology and where they can display the full set of microglial functions which are present only upon interaction with neurons.

IPSC MICROGLIA PROTOCOLS

The first protocols for human embryonic stem cell (hESC)-derived macrophages used cocultures with cell lines, complex cytokine cocktails, and laborious purification steps to enrich for CD34⁺ HSCs and macrophage progenitor colonies [12, 13] and it is unknown if those protocols follow MYP dependent or MYB-independent myeloid differentiation. Later, the James lab developed a simple protocol for the production of macrophages from pluripotent stem cells without the use of additional feeder cells during differentiation, using only interleukin-3 (IL-3) and macrophage colony stimulation factor (M-CSF) to drive myelopoiesis, thereby yielding a pure macrophage population without the need for additional purification steps [14]. They adapted the protocol to feeder-free iPSCs with fully defined conditions and were able to take weekly yields from their macrophage factories for more than 6 months [15]. By showing that the macrophage differentiation of MYB knock out (KO) iPSCs was unchanged, they demonstrated that those cells are MYB-independent yolk sac derived embryonic macrophages, hence their macrophages are indeed ideal microglia progenitors [16]. Furthermore, they showed that in coculture with cortical neurons those cells show

microglia typical ramifications, movement, cytokine release, and transcriptome [17].

Furthermore, protocols for the production of hESC/iPSC-derived microglia have since been developed. Beutner et al. described the production of mouse iPSC microglia emerging from neuronal cultures [18] and Almeida et al. were first to claim they produced human iPSC microglia with an apparently similar protocol, yet methodological details have not been provided [19]. Those microglia have characteristics of an immortalized microglia cell line, which allows fast expansion for large scale experiments. They are not, however, suitable for long-term cocultures and they do not show a convincing microglia transcriptome signature [3]. Schwartz et al. has used human iPSC microglia in three-dimensional (3D) coculture with neurons to study neurotoxicity but a further characterization of the microglia in those brain organoids was regrettably not provided [20]. Several protocols with extensive characterization of human iPSC-derived microglia have been published recently [17, 21–27]. Abud et al. initially use low oxygen during differentiation and need sorting of HSCs before going through a two-step maturation protocol to obtain mature microglia, importantly, they successfully transplanted their iPSC-derived microglia into mouse brains. Takata et al. showed the importance of neuron microglia interaction for the microglial maturation, most of their work, however, was performed with mouse iPSC microglia and only proof of principle data was shown for human iPSC microglia. McQuade from the same lab published a faster protocol using STEMdiff Hematopoietic Kit with confidential composition to obtain HSCs, then they do a fast final differentiation without IL-3 in the medium, those iPSC microglia were also successfully transplanted in mouse brains [26]. Pandya et al. are coculturing iPSC-derived HSCs with astrocytes to obtain iPSC microglia [23]; we assume that this protocol releases microglia most similar to primary microglia released from mixed glial cultures. Ormel et al. found that microglia innately develop within iPSC-derived cerebral organoids and showed that those microglia perform similar as primary human microglia in functional assays [27]. Their cerebral organoid protocol is omitting non-neuronal lineage suppression. From their data, it remains unclear to us if the first microglia progenitors originate within cerebral organoids or invade the cerebral organoids but originate from yolk sac organoids present in a mixed organoid culture, hence, if their results are incompatible with the hypothesis of yolk sac derived origin of microglia that invade the brain. iPSC themselves show relatively high expression of growth factors SCF, VEGF, BMP4, IL34, CSF1, and CSF2 [28], which are used in most of the described iPSC microglia protocols. It was shown previously that adding SCF, VEGF, and BMP4 to initiate HSC differentiation is optional but increases its efficiency [15]. Haenseler et al., who are using the James lab's MYB-independent macrophages, have the simplest and most user-friendly of those protocols [17]. They use only IL-3 and M-CSF to drive myelopoiesis and pure microglia precursors can be collected repeatedly from the macrophage factory supernatants, without the need for any sorting procedure. Those precursors can then be matured to microglia in mono-culture or in coculture with neurons in medium supplemented with IL-34 and GM-CSF. Furthermore, the James lab macrophages are by now the only ones proven to derive MYB-independent by using MYB KO iPSC lines [16]. Haenseler et al. designed coculture conditions for the use as an Alzheimer's disease (AD) model, maturing the iPSC-derived cortical neurons for

65 days after neuronal induction, when they have formed functional synapses and show electrical activity [17, 29] and importantly release all the major β -peptides A β 38/40/42 [30]. Microglia precursors are cocultured for the last 2 weeks of the neuron differentiation and adapt the ramified morphology after approximately 1 week of coculture. Recently, their macrophage precursors were shown to integrate in brain organoids and survive integration into the organoids without supplementation of external growth factors [25]. Xiang et al. use the James lab protocol for production of microglia precursors and then changed to the microglia medium described by Abud et al. for final maturation in mono-cultures [31]; their iPSC microglia show similar expression of key microglia markers as primary microglia, unfortunately a direct comparison to the James lab microglia is not provided.

We conclude that all those protocols provide iPSC-derived microglia, but only direct side-by-side comparisons of the microglia from those protocols could answer which produces the most authentic microglia regarding transcriptome and functional assays. Interestingly, we see in many protocols that iPSC microglia have higher expression of some of the microglia markers than the cultured primary microglia, which might be explained by loss of microglia markers in primary microglia cultures. Although most protocols use IL-3 and M-CSF in their differentiations medium to drive myelopoiesis, it is currently unknown if using additional growth factors improve microglia signature. The optimal choice of growth factors and the presence of serum in the maturation/assay medium should be made dependent on the planned assays. In future protocols, we also expect cocultures with specific neuron subtypes for optimal disease modeling, for example, dopaminergic neurons for Parkinson's disease (PD) models.

DISEASE PHENOTYPING IN DIFFERENT HUMAN MICROGLIA MODELS

The James lab iPSC-derived macrophages/microglia have been used in various disease relevant setups in the James lab or in other labs with minor variations of the protocol. They were used as model for HIV integration [32] and entry [33] and used as a gene therapy model for the primary immunodeficiency chronic granulomatous disease (CGD), where patients lack functional NADPH oxidase, which is responsible for reactive oxygen species (ROS) production [34]. CGD iPSCs were corrected with CRISPR/Cas9 footprintless gene editing [35], which fully restored ROS production in iPSC macrophages. Such patient iPSC lines would be an invaluable tool to investigate effects of partial and full loss of NADPH oxidase function in neuroinflammation, for example, to confirm the results obtained in rat primary neuron-glia cultures, which show neurotoxicity depended on phagocytosis of α -synuclein and activation of NADPH oxidase with production of ROS [36]. The James lab iPSC macrophages were used for a transcriptional analysis comparing lipopolysaccharide (LPS) induced inflammation between peripheral blood mononuclear cell-derived macrophages and iPSC-derived macrophages [37] and to assay the heterogeneity of LPS induced inflammatory response of iPSC macrophages with single cell RNA sequencing [38]. Furthermore, they were compared with primary mouse macrophages for *Escherichia coli* phagocytosis [39] and were used to elucidate the role of FRMD8 in tumor necrosis factor α (TNF- α) shedding pathway [40]. Neurological disease relevant phenotypes have been described in these iPSC macrophages from patients with Gaucher's disease

with GBA mutations, where reduced ROS production and impaired chemotaxis was observed [41, 42]. In PD patients with synuclein alpha (*SNCA*) triplication, the macrophages showed accumulation of α -synuclein and reduced phagocytosis [43]. LRRK2 mutant macrophages showed alterations in *Mycobacterium tuberculosis* infection [44] and TREM2 mutants showed defects in TREM2 processing [25] and defects in phagocytosis of apoptotic neurons [45]. Interestingly, TREM2 with the R47H mutation is aberrantly spliced only in mice but not human primary- and iPSC-microglia [31].

The Neumann lab showed reduced progranulin secretion in iPSC-derived microglia from a frontotemporal dementia (FTD) patient [46]. APOE4 variant of mono-culture microglia, differentiated following the Muffat protocol, have been shown to have impaired amyloid- β 42 clearance [47]. Although in a human tri-culture system with SV40 immortalized microglia, iPSC astrocytes and iPSC neurons, activation in response to Swedish mutant β -amyloid precursor protein (APP) neurons, which secrete high levels of A β , was demonstrated [48].

Independent of which differentiation protocol is used, one must distinguish between mono-culture, two-dimensional (2D) neuron coculture protocols, and 3D culture/brain organoids. An overview of advantages and disadvantages of those models and published as well as suggested disease phenotyping assays is given (Table 1). Depending on the research question, cocultures indeed can be mixes of iPSC-derived cell-types and human or animal primary cells or cell-lines, this can also include the transplantation of human iPSC microglia to animal models. For preclinical testing of antineurodegeneration drugs, it is also crucial to show that the drug can cross the BBB. These tests are currently best done in animal models, however, human primary cell [51] and iPSC BBB organoid [50] models have been developed.


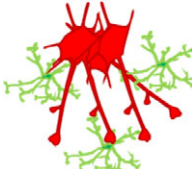
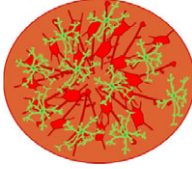
GENETIC ENGINEERING OF IPSC

Perhaps one of the bigger advantages of iPSCs is that they can be cultured long-term without loss of genome integrity and pluripotency. Patient iPSCs are, therefore, a theoretically unlimited source of material for disease modeling or cell replacement therapies. iPSCs are well accessible for genetic manipulations whereas postmanipulation clonal lines can be selected and quality controlled before differentiation. Retroviruses and adeno-associated viruses (AAV) are a good tool for the correction of loss of function mutations by overexpression of the malfunctioning protein when correct gene dosage is irrelevant [52, 53].

CRISPR/Cas9, Zinc finger, and TALEN technology can be used for gene editing to introduce a transgene to a defined place in the genome. Introducing the transgenes to save harbor sites such as AAVS1 leads to phenotypic correction [49]. But any of those technologies can also be used to create gene knockout lines or gene correction by directly targeting the gene of interest. CRISPR/Cas9 was used for footprintless gene correction [35] and tagging of endogenously expressed genes is possible [54]. It has also been used in patient iPSCs to remove additional gene copies such as the very rare *SNCA* gene triplication [55], which causes early onset PD and to remove surplus GGGGCC repeats in *C9orf72* that cause amyotrophic lateral sclerosis (ALS) [56].

Reliable disease phenotyping can be carried out with several patients who carry the same mutation but who are not siblings and, therefore, have different genetic backgrounds. Alternatively, an isogenic pair can be created either by

Table 1. Comparison of different iPSC microglia models

Model	Advantages (+)/limitations of the model (–)	Published disease phenotyping/suggested phenotyping assays
Microglia Mono-culture 	+ Monocultures are suitable to study intrinsic defects of the microglia + Scalable for high throughput screens + Cell lysis for protein and RNA isolation directly in well – Many microglia functions are related to interactions with neurons and, therefore, can exclusively be observed in cocultures	Reduced progranulin secretion in microglia of FTD patients [46] SNCA triplication with reduced phagocytosis of zymosan and upregulation of cytokines/chemokines [43] APOE4 with reduced uptake of amyloid- β 42 [47] GBA mutants with reduced ROS production and impaired chemotaxis [41, 42] Restored macrophage function after gene therapy [35, 49] TREM2 T66M, W50C mutants with reduced phagocytosis of apoptotic neurons [45] and R47H showed altered TREM2 splicing in mouse but not human microglia [31]
Microglia/neuron Coculture 2D 	+ Microglial scanning of neuronal environment observed + Transcriptome closer to primary microglia + Focus on key players of neurodegeneration – Additional cell types are involved in neurodegeneration (astrocytes, oligodendrocytes) – Protein and RNA isolation needs elaborate separation of microglia and neurons (MACS, FACS)	Microglia morphology (ramified vs. amoeboid), microglia migration and Inflammatory response in coculture, which is not sum of mono-cultures [17] Microglia induced neurotoxicity Microglia induced synapse loss Microglial clearance of neuron derived toxic products such as amyloid- β and α -synuclein
Microglia addition to 3D cultures and brain organoids 	+ Authentic cell interactions + Triculture system is well defined + Triculture system can be imaged in 96-well plate + Cerebral organoids can include BBB models [50] – Long maturation times of organoids – Organoids are hard to image – Low throughput – Inguided heterogenous brain organoid are variable in cell composition (reproducibility)	TREM2 mutant microglia have unchanged invasion of brain organoids [25] Microglia activation by Swedish APP neurons [48] Microglia migration/integration into brain Complex interactions of different brain cell types astrocyte/microglia/neuron/oligodendrocyte crosstalk upon stress/damage Microglia proliferation/microgliosis and astroglyosis upon stress/damage Microglia related BBB damage

Abbreviations: iPSC, induced pluripotent stem cell; FTD, frontotemporal dementia; BBB, blood–brain barrier.

correction of a patient iPSC line to obtain an isogenic control line, or by introducing the patient mutation into a healthy control line to reproduce the rare patient phenotype (Fig. 1A). Using isogenic pairs has the big advantage that two lines are the minimal requirement for an experiment. Working with sporadic disease patient, iPSCs are perhaps the most challenging, as heterogenous phenotypes are expected and high patient numbers are needed to be able to stratify patients postanalysis (sex, age, biomarker, severity of the disease, look for compound heterozygous SNPs, etc.).

Reporter iPSC lines have many possible applications in monocultures and in the coculture system (Fig. 1B). Retroviruses can be used to create reporter lines that express fluorescent proteins under specific promoters to identify cells of interest, for example, thyroxine hydroxylase as a marker of dopaminergic neurons [57], or nuclear factor κ B (NF- κ B) to identify activated microglia [58]. The expression of tagged proteins allows easy visualization and tracking of the protein of interest in live cells. However, retrovirally introduced genes are often silenced in iPSCs or during differentiation to the cell of interest. This can be prevented by using silencing resistant promoters to drive transgene expression, with a promoter combined with sequences that prevent silencing such as the UCOE sequence [52, 59] or by introducing a selection cassette and apply constant selection pressure (such as puromycin, neomycin, zeocin) as previously done for RFP iPSC-derived microglia before adding them to the neuronal cocultures [17]. Another

option is to gene-edit the endogenous gene locus to coexpress a fluorescent reporter or directly introduce a tag such as the short FLAG tag to visualize the endogenous protein [60]. Furthermore, modeling tools are inducible promoters allowing to switch genes on or off in the differentiated cells [61].

USING MICROGLIA/NEURON COCULTURES FOR DISEASE PHENOTYPING AND DRUG SCREENING

Recently, it was demonstrated in a mouse model that neurodegeneration through synapse loss could occur even if the neurons were kept unperturbed but by removing a gene (in this case, *TARDP/TDP-43*) specifically in microglia suggesting a key role for microglia in neurodegeneration and thus emphasizing the need for human iPSC microglia–neuron cocultures to study how this synapse loss could be prevented in patients [62].

A screen for drugs that prevent synapse loss in the iPSC coculture system could be based on reporter lines. Neurons with fluorescently tagged synapses could be used to assay synaptotoxicity combined with microglia containing an activation reporter. As a first step, one would have to identify synaptotoxic conditions to recapitulate microglia-induced synapse loss in the cocultures. The second step would be to screen for reagents that interrupt the microglia/neuron interaction leading to the induced synaptotoxicity and stop the synapse loss. Such a coculture system combined with fully automated high-

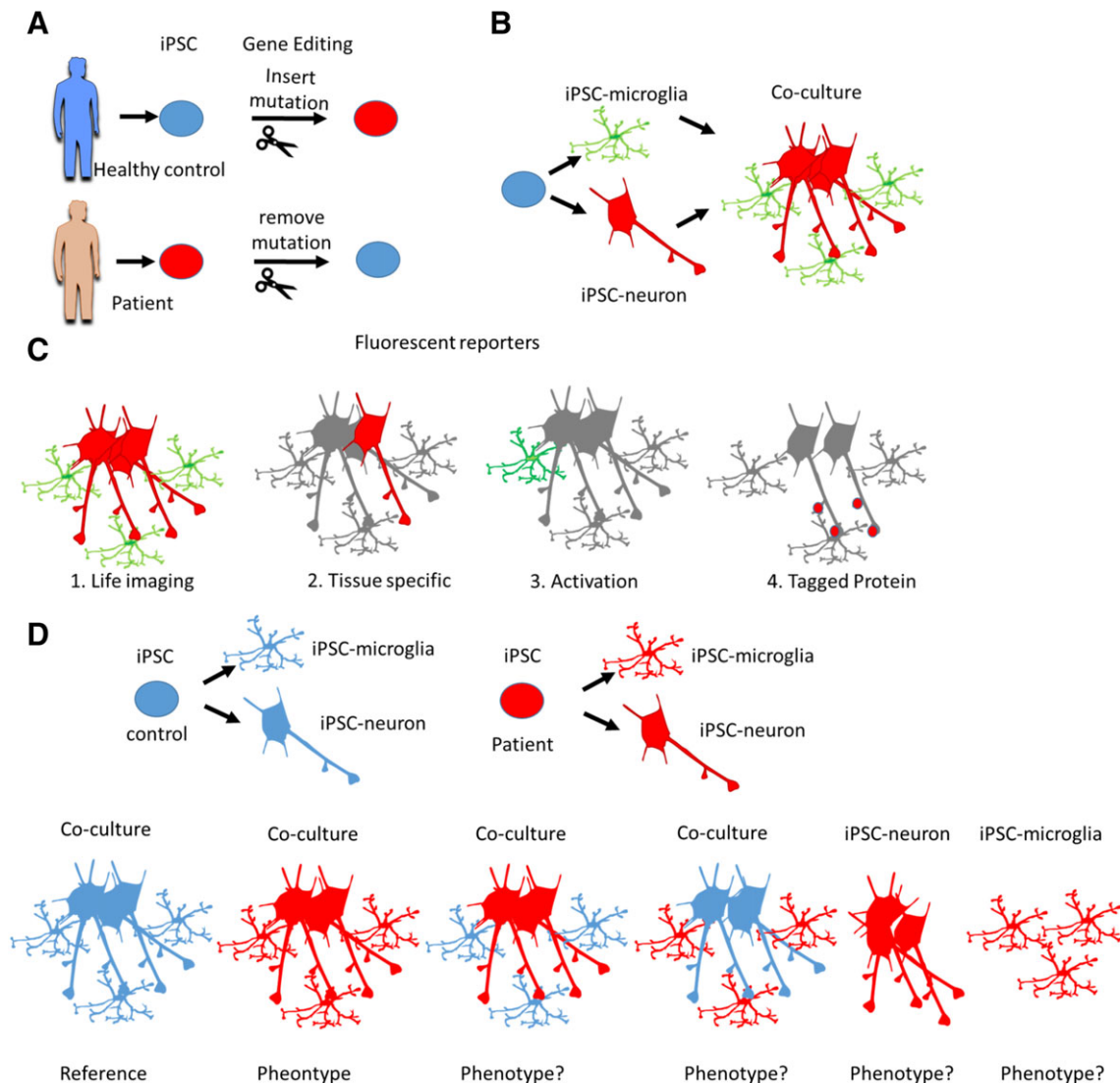


Figure 1. Induced pluripotent stem cell (iPSC) tools and their suggested use in the microglia/neuron coculture model. **(A):** iPSCs derivation from healthy controls or patients. On iPSC level, mutations can be introduced to healthy control cells or removed from patient cells without otherwise changing the genetic background of the iPSC line, thus obtaining ideal isogenic control lines. **(B):** Examples for the use of fluorescent reporters in iPSC microglia/neuron cocultures. **(B1):** Neurons (red) and microglia (green), which express cytosolic fluorescent proteins allow the identification of corresponding cells in life imaging experiments. **(B2):** Tissue-specific expression of fluorescent proteins can be used to identify subtypes of cells in the culture (e.g., TH for dopaminergic neurons). **(B3):** Tissue-specific expression of fluorescent proteins can show activation status of microglia (e.g., NF- κ B). **(B4):** Tagged proteins can show the position of a protein (e.g., PSD95 to mark the synapses). **(C):** Strategy to identify the disease-causing cell type by combining matched and mismatched control and patient neurons/microglia and comparison to mono-cultures to test if cells produce the respective phenotype on their own.

content-imaging and automated image analysis would allow for high-throughput drug screens.

Probably, more relevant for drug screens are patient/gene-engineered iPSC microglia/neuron cocultures, where the first step is to identify a well-reproducible disease phenotype. Ideally, one can confirm that this phenotype is resolved in isogenic controls/or by using other gene therapy approaches. Once a phenotype is identified, the system allows dissecting to understand which cells are the origins of the phenotype and, therefore, contain the potential drug target. This dissection can be done with mono-cultures of neurons and microglia but also by match/mismatch experiments with healthy control and disease microglia and neurons in a coculture system (Fig. 1C). Once phenotypes are identified, the system is ideal for drug screening.

Furthermore, patients could be stratified according to phenotypes, and the response to the drugs could be tested in subgroups or in a real personalized medicine approach.

Match/mismatch experiments would also answer which cells have to be targeted by cell replacement therapies or by gene therapy approaches.

POTENTIAL CLINICAL USE OF GENE-EDITED iPSC

Gene therapy approaches to treat neurodegenerative diseases typically use AAV vectors that are injected directly into the brain [53,63,64]. For example, an AAV9 gene therapy vector successfully corrected Gaucher's disease phenotypes in fetal

and newborn GBA KO mice and has been demonstrated to be able to transduce neurons in a macaque brain after in utero delivery of the gene therapy vector [53].

Implanting autologous gene-edited iPSC-derived cells into patients open new therapeutic possibilities. Human iPSC-derived dopaminergic neurons were recently shown to have great therapeutic benefit as cell-based therapeutics in a primate model of PD [65]. The tools to edit-iPSC are there, as we know iPSC-derived microglia successfully integrated in murine brains [21,26] and GMP grade iPSC-derived HSCs with the needed quality controls are under development [66].

The obvious gene therapy approach is to correct the patient iPSC lines back to a healthy gene variant. But one could also go a step further and create enhanced iPSC-derived cells, for example, with improved clearance for specific toxic peptides or reduced neurotoxic repertoire. Our own lab results show that tweaking a gene in microglia could alter the outcomes that result in neurodegeneration [50] and thus it is totally conceivable to alter iPSC-derived microglia to have properties that can be exploited therapeutically for neuroprotection.

CONCLUSION

Recently, several convincing protocols for the generation of human iPSC-derived microglia have been published [17,21–24]. The MYB-independent ontogeny of those microglia has so far only been convincingly demonstrated by the protocol developed in the James lab which uses MYB KO iPSCs [16]. The most authentic microglia morphologies were observed in cocultures with neurons, respectively, after injection into mouse brains, which also correlated with the most microglia like transcriptome [17,24]. However, most disease relevant phenotyping is so far done in mono-culture microglia. For many phenotyping assays, mono-cultures are an acceptable and economically plausible choice, but to investigate the role of microglia interactions with neurons in neurodegenerative diseases, the coculture of microglia

with neurons is a prerequisite. There are now several models of 2D and 3D coculture available that would allow assaying neurotoxicity at different levels of complexity. The major advantages of iPSC-derived microglia as compared with primary human cells are the theoretically unlimited availability of patient material as well as the possibility to create isogenic controls and to engineer reporter cells designed specifically to your assay readouts. Match/mismatch experiments can identify the origin of neurotoxicity and thus tells us which cell type has to be targeted by drugs, gene therapy, or cell replacement therapies. AD, PD, and FTD/ALS are all complex and heterogeneous diseases where patients stratify in drug responses. Drug candidate testing in iPSC cocultures using cells from big patient cohorts promise to stratify the patients according to their drug responses. But we also foresee a future where complex coculture systems are used in a real personalized medicine approach to test the drug response of each individual patient before onset of treatment.

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AUTHOR CONTRIBUTIONS

W.H.: conception and design, collection and assembly of data, manuscript writing; L.R.: conception and design, financial support, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

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